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Hepatitis E Virus: First Description in a Pet House Rabbit. A New Transmission Route for Human?
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Summary
In this work, we identified for the first time hepatitis E virus (HEV) in a pet house rabbit, an adult 7 years old female of domestic rabbit (Oryctolagus cuniculus). Importantly, the resulting phylogenetic tree showed that the HEV strain identified in the pet house rabbit was closely related to a human HEV sequence; this finding reawakens concerns regarding the zoonotic risk represented by HEV in animals and expands to house rabbit the spectrum of potential source of infection for humans. Potential for domestic transmission of HEV to humans should be taken into account.

Introduction
Hepatitis E virus (HEV), the aetiological agent of hepatitis E, belongs to the family Hepeviridae which, according to a recent classification, is divided into the genera Orthohepevirus (all mammalian and avian HEV isolates) and Piscihepevirus (cutthroat trout virus). Species within the genus Orthohepevirus are designated Orthohepevirus A (isolates from human, pig, wild boar, deer, mongoose, rabbit and camel), Orthohepevirus B (isolates from chicken), Orthohepevirus C (isolates from rat, greater bandicoot, Asian musk shrew, ferret and mink) and Orthohepevirus D (isolates from bat) (Smith et al., 2014).

Materials and Methods
Bacteriological analyses were carried out to determine the possible cause of death following standard procedures. Rotavirus, RHDV and MV were considered and investigated by indirect immunofluorescence (MV) and by PCR (RHDV and Rotavirus). For histological examination, samples were fixed in 10% neutral buffered formalin (pH7) and paraffin-embedded for histological examinations. Four-micrometre sections were cut using a microtome and stained with haematoxylin and eosin.

For HEV detection, total RNA was extracted from 50 to 100 mg of liver samples and 350 ll of whole blood samples with Trizol RNA isolation reagents (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions. Three microlitres of extracted RNA from liver and whole blood samples was tested by a one-step real-time RT-PCR assay, targeting a highly conserved region of ORF3 according to Jothikumar et al. (2006). The 50 region of the
ORF2 gene, coding for the putative capsid polypeptide, was selected for phylogenetic analysis; for this purpose, a nested PCR assay was performed (Huang et al., 2002). Amplification products were sequenced using the Big Dye Terminator v3.1 chemistry on an ABI 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s protocol. The newly generated rabbit sequence and HEV sequences available in GenBank were aligned using BioEdit (Ibis Biosciences, Carlsbad, CA, USA) software and the CLUSTALW algorithm. Nucleotide substitution models were evaluated using jModelTest2 (Free Software Foundation, Boston, MA, USA), and the best model was selected according to the AIC analysis. Phylogenetic analysis was performed using MEGA version 6 (Tamura et al., 2013), and tree was constructed according to the maximum likelihood criterion. The nucleotide substitution model was set according to jModelTest2 output and was general time reversible (GTR) with gamma (G) distribution. Statistical robustness and reliability of the branching order was tested by bootstrap analysis using 1000 reiterations.

Results
At the external examination the rabbit appeared in good body condition, with blood spots surrounding nose and mouth. The macroscopic evaluation revealed haemoperitoneum, enlargement of the uterus and haemorrhagic lungs. Moreover, the liver appeared increased in volume and consistence, pale and with evident texture. Histologically, diffuse oedema and focal peribronchial lymphocytic infiltrate were observed in the lungs; spleen showed a severe lymphocytic depletion and liver appeared autolytic. No lesions were present in heart and kidney. Investigation for MV, RHDV and Rotavirus resulted negative. Rabbit liver tested positive by real-time PCR positive. The result was confirmed by nested PCR for HEV characterization. Determination of sequence similarity based on nucleotide homology revealed that the analysed ORF2 region of the rabbit strain shares a variable degree of nucleotide sequence identity with HEV sequences from different host species available in GenBank: 82.6–90.1% with rabbit HEV; 73.0–80.9% with swine HEV; 74.3–81.6% with wild boar HEV; 71.4–89.5% with human HEV; and 77.3% with one HEV sequence from wild deer. The phylogenetic tree based on the aligned nucleotide sequences of the 50 region of HEV ORF3 is shown in Fig. 1.

Discussion
Although necropsy and laboratory investigation revealed that HEV was not the cause of death, because of a Pasteurella spp. was isolated from lungs, we report the first detection of the virus in a pet house rabbit. As rabbit HEV is a novel virus, more investigations are needed to clarify its role in rabbit health, as well as its contribution to the transmission of hepatitis E in humans. Cossaboom et al. (2011) reported the presence of HEV in farmed rabbits in Virginia (USA), in 14 (16.5%) of 85 serum samples and 13 (15.3%) of 85 faecal samples, while seroprevalence was 36% (16 of 85 serum samples). Sequence analyses of the samples showed that HEV from rabbits was closely related to genotype 3. In France, HEV RNA was found in 7.0% of the farmed rabbits, and in 23% of the wild rabbits, a relationship between HEV RNA detection and age has been hypothesized but not assessed due to the lack of a designed study. In that study, phylogenetic analysis indicated that
the genomes of rabbit HEV strains from China and France or TLS-18516-human were <80% identical with HEV genotype 3, regardless of which method was used to align the sequences, compatible with the definition of a new genotype. Interestingly, comparison with HEV sequences of human strains and reference sequences identified a human strain (TLS-18516) closely related to rabbit strain HEV (Izopet et al., 2012).

In our report, the identified pet house rabbit HEV strain was characterized by phylogenetic analysis using the maximum likelihood method on a variable region of 50 end of the ORF2 gene, which provides a phylogenetic signal comparable to full genome analysis of HEV (Lu et al., 2006). Most notably, the resulting phylogenetic tree showed that the HEV strain identified in the pet house rabbit and the human TLS-18516 strain group together into an isolated monophyletic branch supported by 90% bootstrap confidence value within the rabbit HEV clade (Fig. 1). This finding reawakens concerns regarding the zoonotic risk represented by HEV in animals and expands to house rabbit the spectrum of potential source of infection for humans. In this report, we carried out a comprehensive phylogenetic analysis, by including all homologous rabbit HEV sequences available in GenBank at the moment of writing, together with representative sequences of HEV genotypes and subtypes from different hosts. The results show that rabbit HEV strains form a clade well distinct from other HEV genotypes leaning towards the classification of rabbit HEV as a new genotype, as proposed in previous studies (Zhao et al., 2009; Geng et al., 2011; Lhomme et al., 2013).

In Italy, no data were available about circulation of HEV both in farmed and pet house rabbits. In north-western Italy, HEV has been demonstrated to circulate in swine and wild boar populations in previous investigations by our group (S. Peletto, A. Rosamilia, C. Caruso, N. Vitale, L. Chiavacci, P. Modesto, C. Boin, P. L. Acutis, E. Messana, E. Gobbi, S. Origlia, B. Sona, L. Masoero, unpublished; Caruso et al., 2014). However, both swine and wild boars HEV strains isolated in the same geographical area, clustered together with reference HEV genotype 3 strains (Fig. 1) and were identified as subtypes f, c and e, thus leading to exclude that cross-species transmission occurred between Suidae and the house rabbit. On the basis of collected anamnestic data, the rabbit may have become infected with HEV in several ways; consumption of contaminated domestic food (vegetables and fruit) as well as contaminated water may have represented a transmission route. Nevertheless, hay provided to pet owner by an organic farm could also be take into account as primary source of infection. Another hypothesis is that, as the rabbit lived in an external environment (garden) only in the summer season, it might have come in direct contact with infected wild rabbits, which are known to be a potential reservoir of HEV circulation. This latter hypothesis would deserve further investigation since, by date, no data are published about HEV prevalence in wild rabbits in north-western Italy. Importantly, our main findings are that for the first time, we identified circulation of HEV in a pet house rabbit; this result could be relevant as it could arise the concern for a novel domestic transmission route for human. In addition, phylogenetic analysis showed that rabbit HEV strain isolated in this work is very closely related to
human HEV isolated in France. This is very interesting as HEV is transmitted mainly through the faecal–oral route due to faecal contamination of drinking water; nevertheless, viraemia and, consequently, shedding of viral particles in faeces increases the risk of potential zoonotic transmission by a direct way.

Over the last decade, the number of rabbits kept as pets has increased dramatically. The 2009–2010 Pet Owners Survey carried out by the American Pet Products Association 2009–2010 Pet Owners Survey also confirmed that rabbits are the most commonly kept small animal pet in the United States. The results of our work indicate that house rabbit could be a reservoir for HEV infection and could play an important role in the zoonotic transmission, above all for children who frequently come in close contact with these pets. Awareness by the veterinary practitioner and above all, the knowledge of good hygiene practice for the pet owner is crucial in the prevention of zoonotic diseases.

However, even if the true potential of cross-species HEV transmission from rabbits to humans is not known and further issues remain to be clarified, our data proved that also domestic rabbit should be considered in the epidemiological scenario of HEV infection.

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Conflict of Interest Statement
None of the authors of this manuscript has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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